CHEMICAL RESEARCH, DEVELOPMENT & ENGINEERING CENTER

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IN VITRO SYSTEM FOR THE ASSESSMENT OF THE POTENTIAL ANTIFERTILITY AFFECT OF CHEMICALS

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The medium and culture conditions that support rabbit sperm cell motility and development of hyperactive motion were determined. Sperm motility was excellent when cultured in defined medium (DM) supplemented with heat inactivated rabbit, calf, or horse serum; but cell viability was poor. Cell viability was improved in DM alone or in Biggers, Whittingham, and Whitten medium but was best when incubated in Toyoda and Chang or T6 mediums. Hyperactive motion developed in the four media when incubation was carried out at 37 °C in 95% air and 5% CO^2. A preincubation step was not necessary. Sperm cell viability and hyperactive motility development was rabbit specific.

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PREFACE

The work described in this report was authorized under Project No. F8J2-10-005. This work was started in February 1989 and completed in September 1990.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," National Institute of Health Publication No. 85-23, 1985, as promulgated by the committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council, Commission of Life Sciences (Washington, DC). These investigations were also performed in accordance with the requirements of AR 70-18, "Laboratory Animals, Procurement, Transportation, Use, Care, and Public Affairs."

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IN VITRO SYSTEM FOR THE ASSESSMENT OF THE POTENTIAL ANTIFERTILITY AFFECT OF CHEMICALS

1. INTRODUCTION

Rabbit sperm cells are commonly capacitated in defined medium (DM).[1] In studies to validate the CellSoft Motion Analysis System and to determine its use for toxicological testing,[2,3] we found that after 4 to 5 hr of incubation in DM under the conditions described by Brackett and Oliphant, the percentage of motile rabbit sperm routinely decreased to only 50-90% of the initial value. This was accompanied by a drop in the quality of sperm motility, and few hyperactive sperm cells, an indication of the capacitation status of sperm cells, were seen. The decline in the quality of motility with incubation time varied among sperm cells from different rabbits but was relatively constant within a rabbit. The key steps in the capacitation of rabbit sperm cells, as described by Brackett and Oliphant,[1] are preincubation of washed cells in high ionic strength (HS) medium followed by incubation at 37 °C in DM under an atmosphere of 8% O_2, 5% CO_2, and 87% N_2. It is unclear if the decreases in the numbers of motile sperm cells and in the quality of sperm movement are due to some deficiency in the medium, the conditions of incubation, or is an intrinsic property of the sperm cells of individual rabbits. An in vitro system for the assessment of chemical toxicity, that is based on measurements of changes in motion characteristics of sperm cells induced by the chemical, must be able to support sperm motility for an extended period. Alterations in culture conditions and medium composition were made in attempts to improve sperm cell viability and obtain an environment suitable for the development of hyperactive motility. The ultimate goal was to develop an in vitro system to assess the adverse fertility and cytotoxic effects of xenobiotics. These experiments are described in this report.

2. MATERIALS AND METHODS

2.1 Animals.

New Zealand white rabbits were individually housed in standard rabbit cages in a room maintained at 25 ± 3 °C and 50 ± 10% relative humidity (RH) with a 12 hr light/dark cycle. Standard approved laboratory rabbit chow and water were available ad libitum.

2.2 Collection and Purification of Sperm Cells.

Sperm cells were collected and purified by centrifugation through a discontinuous Percoll gradient as previously described.[4]

2.3 Incubation and Videotaping of Sperm Cells.

Sperm cells suspended at a concentration of 1 to 2 x 10^7/mL in the medium under study were incubated at 37 °C under an atmosphere of either 5% CO_2, 8% O_2, and 87% N_2 or 5% CO_2 and 95% air. Sperm motion was assessed under negative phase contrast at 100X. Videotaping and analysis of sperm motion characteristics with the CellSoft system were carried out as described previously.[2,3]

For preincubation studies, one-half of the rabbit semen was washed with HS medium, preincubated for 15 min in HS medium, and washed with DM before resuspension in DM either immediately or after centrifugation through a Percoll gradient[2] to remove seminal fluid contaminants. The other half of the semen was treated identically except that washings and preincubation were carried out only in DM. In other experiments, preincubation in either DM or HS medium and resuspension in DM were carried out after centrifugation of semen through the Percoll gradient. The quality of sperm motion was assessed after incubation for 1, 3, 5, and 20-22 hr. Each treatment regimen was carried out with semen from 2 to 3 rabbits.

For gas mixture studies, one-half of a sperm suspension, previously centrifuged through a Percoll gradient and preincubated in HS medium, DM, or not preincubated in either medium, was incubated in an atmosphere of 5% CO_2 and 95% air; the other half was incubated in an atmosphere of 8% O_2, 5% CO_2, and 87% N_2. Sperm motility was observed at 1, 3, 5 and 22 hr. Each experiment was repeated with sperm from 2 to 3 rabbits.

The affect of sera on motility was studied with sperm cells that were purified by centrifugation through a Percoll gradient. Purified cells were resuspended in either DM or DM containing 20% heat inactivated rabbit, horse, or calf serum and incubated in an atmosphere of 5% CO_2, 95% air at 37 °C, and sperm motion was recorded on videotape at 1, 3, and 5 hr.

The affect of medium composition on sperm motility was investigated with sperm purified by washing with DM or centrifugation through a Percoll gradient. Purified sperm cells from one rabbit were divided into four parts, and each part was resuspended in one of the four test mediums. Cell suspensions were incubated in 5% CO_2, 95% air at 37 °C, and motility changes were observed at 3, 5, and 22 hr. Experiments were repeated with sperm from several rabbits.

2.4 Sera.

Rabbit, horse, and calf sera were obtained from Gibco Laboratories, Grand Island, NY. Sera were inactivated by heating at 56 °C for 1 hr and stored frozen at -10 °C. Sera were also

inactivated by heating briefly to 85 °C and centrifugation for 15 min at 10,000 *g* at 4 °C to remove the denatured protein. The supernatant was stored at -10 °C.

3. RESULTS

3.1 Preincubation Conditions.

The necessity for incubating sperm cells in HS medium was investigated by experiments in which both halves of a semen sample from a rabbit were subjected to identical treatments except for exposure to HS medium. As observed previously,[2,3] the percentage of motile sperm was high, and the quality of sperm motion was good at 3 hr; however, this was seldom the case at 5 hr. Sperm from some rabbits was immotile after 5 hr of incubation; but, a low percentage (1-5%) from others was still motile at 22 hr. An occasional sperm with hyperactive motion was seen in sperm suspensions from some rabbits after 1, 3, or 5 hr of incubation; but, most of the sperm cells that were motile at 22 hr possessed hyperactive motility.

Sperm cells from the one semen sample behaved similarly, irrespective of the preincubation treatment. Thus, if sperm cells preincubated in HS medium were motile and hyperactive at 22 hr, cells of the same semen sample preincubated in DM were also hyperactive at 22 hr. Sperm motility behavior was relatively constant within rabbits but was quite different among the 15-18 rabbits of the colony. For example, hyperactive motion was never observed after 22 hr of incubation in sperm cells from some rabbits but was almost always present in sperm suspensions obtained from others. Sperm from some rabbits remained highly motile after 5 hr of incubation, while few sperm cells from other rabbits were motile after 5 hr. These results show that the quality of sperm motion is independent of preincubation conditions but is an intrinsic property of the cells from individual rabbits.

3.2 Composition of Gas Mixture.

Sperm cells of other species are routinely cultured in an atmosphere of 5% CO_2 and 95% air rather than the low (8%) O_2 atmosphere used by Brackett and Oliphant.[1] The affect of these two gas mixtures on sperm motility and viability was compared. As previously observed, the quality of sperm motion was generally poor after 5 hr of incubation, and few sperm cells were motile after 22 hr. Most sperm cells that were motile at 22 hr possessed hyperactive motility. The motility characteristics of sperm cells from the one suspension were similar whether incubated in the presence of either one or the other gas mixture. The characteristics of sperm cell motion were rabbit specific and independent of the composition of the gas mixture used for incubation.

3.3 Medium Composition.

Rabbit serum has been reported to enhance rabbit sperm cell capacitation.[5,6] To investigate if rabbit, horse, or calf serum either accelerates the acquisition of hyperactive motion or improves the viability of rabbit sperm cells, DM containing sera in place of bovine serum albumin was used for culture. Sperm cells formed aggregates in the presence of all three sera; aggregation was least in horse and worse in calf sera. All three sera induced an erratic mode of motion in a subpopulation of cells characterized by spurts of forward progression, a pause, and then progression in a different direction. Other subpopulations of sperm cells swam in a random nonprogressive manner with an exaggerated side-to-side head movement observed frequently in populations of capacitated sperm cells. This subpopulation of sperm cells appeared earlier and/or in greater numbers in sperm suspensions incubated in DM with sera than in control suspensions incubated in DM. However, after 5 hr of incubation, the two subpopulations of sperm cells were absent or greatly reduced in number, and there was usually little difference between the motion of sperm cells incubated in DM plus sera and control sperm cells. The percentage of motile sperm cells was also lower than at earlier times. Sperm cells from some rabbits were immotile by 5 hr. Sperm cells incubated in sera generally were immotile after 22 hr of incubation. Again, sperm motility behavior was rabbit specific.

Analysis of sperm motility at 1, 3, and 5 hr by the CellSoft system showed that there was no consistent difference in curvilinear velocity (Vc) between control DM and sperm incubated DM plus sera, unless incubation was carried out in rabbit serum inactivated at 85 °C (Table 1). In this instance, the velocity was significantly higher (two sample t-test) in the control sperm population than in the population exposed to rabbit serum. On the other hand, the linearity (Lin) of sperm incubated in the presence of rabbit, calf, or horse serum was significantly lower, 21 times out of 44 tests (two sample t-test), than control cells for all the rabbits at the 1-, 3-, and 5-hr intervals. The average amplitude of lateral head displacement of sperm cells incubated in DM plus serum was also higher than the control in several instances. These results are consistent with the early appearance and larger subpopulation of sperm cells with random nonprogressive motility and large side-to side head movement, when sperm cells were cultured in DM plus sera.

Table 1. Affect of Adding Sperm to DM on Rabbit Sperm Cell Motility Parameters

Rabbit	Time (h)	Serum	% Motile	Vc	Lin	AALH	BCF
179	1	Rabbit	*	o	o	o	o
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		+	-	-	-	-
336	1	Rabbit	#	-	-	o	o
	3		#	-	-	o	o
	5		#	-	-	o	o
	22		#	-	-	-	-
263	1	Rabbit	-	o	-	h	-
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		-	-	-	-	-
421	1	Rabbit	-	-	-	o	o
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		-	-	-	-	-
465$	1	Rabbit	-	-	-	o	-
	3		-	-	-	o	o
	5		-	-	-	o	o
	22		@	-	-	-	-
510	1	Horse	-	o	-	h	-
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		-	-	-	-	-
485	1		-	h	-	h	-
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		-	-	-	-	-
341$	1		-	o	-	h	-
	3		-	o	o	h	o
	5		-	o	o	h	o
	22		-	-	-	-	-
556	1		-	o	-	o	-
	3		-	o	o	o	o
	5		-	o	o	o	o
	22		-	-	-	-	-
556	1	Calf	-	o	-	h	-
	3		-	o	-	h	-
	5		-	-	-	-	-
	22		-	-	-	-	-
197$	1		-	o	-	h	-
	3		-	o	-	h	-
	5		-	h	-	h	-
	22		-	-	-	-	-

One half of a rabbit sperm cell preparation was incubated in DM and the other half in DM containing 20% serum. Motility patterns were videotaped at 1, 3, and 5 hours and analyzed by the CellSoft system. Differences between the values of the motion parameters in the two sperm cell suspensions were assessed by the two sample t-test.

* significantly lower
- not significantly different
+ motile at 22 hours
# significantly higher
@ not motile at 22 hours
$ serum heated to 85°C

3.4 Alternate Medium for Culture.

The ability of the medium of Biggers, Whittingham and Whitten (BWW),[7] Toyoda and Chang (TOY),[8] or T6 medium[9] to support rabbit sperm cell motility and the acquisition of hyperactive motion were investigated because of the failure of DM plus sera to improve sperm viability. Within a rabbit, there appeared to be little difference, over a 5 hr incubation period, in the

percentage of motile cells and the quality of sperm motion when sperm suspensions were incubated in the different media. In one videotaped experiment (rabbit 338) in which sperm motion was analyzed by the CellSoft system, no significant differences (two sample t-test) were found in motility parameters between sperm cells incubated in DM and BWW, T6, or TOY. Sperm cells from virtually all rabbits used in this series of experiments were motile after 22 hr of incubation in all media, but the percentage of motile cells were higher in cell suspensions incubated in T6 and TOY. Small numbers of sperm cells swimming with a "jerky" and/or vigorous nonprogressive motion appeared in the sperm suspensions of several of the rabbits. In general, these subpopulations appeared earlier, were larger in size, and were present after 22 hr if sperm suspensions were incubated in either T6 or TOY rather than in BWW or DM (Table 2). Most sperm that were motile at 22 hr moved with a jerky or nonprogressive motion.

Table 2. Affect of Incubation Medium on Rabbit Sperm Cell Motion

Rabbit	Time (Hr)	Motile				Motion: "Jerky"				Motion: Non-Progressive			
		DM	BWW	T6	TOY	DM	BWW	T6	TOY	DM	BWW	T6	TOY
102	3	+	+	+	+	-	-	+	-	-	-	-	-
	5	+	+	+	+	-	-	+	-	-	-	+	-
	22	-	+	+	+	-	-	-	-	-	-	-	-
564	3	+	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	-	-	+	+	-	-	+	+
	22	+	+	+	+	-	-	+	+	-	-	+	+
557	3	++	++	++	++	++	++	++	++	++	++	++	++
	5	++	++	++	++	++	++	++	++	+	++	++	++
	22	+	+	+	+	+	+	+	+	+	+	+	+
425	3	+	+	+	+	+	+	+	+	-	-	-	-
	5	+	+	+	+	+	+	+	+	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	-	-	-
510	3	+		+		-		-		-		-	
	5	+		++		-		++		-		-	
	22	+		+		-		+		-		+	
421	1	+	+	+	+	-	-	-	-	-	-	-	-
	3	+	+	+	+	-	-	-	-	-	-	-	-
	5	+	+	+	+	-	-	-	-	-	-	-	-
	22	+	+	+	+	-	-	-	-	-	-	-	-
102	1	+	+	+	+	-	-	-	-	-	-	-	-
	3	+	+	+	+	-	-	-	-	-	-	-	-
	5	+	+	+	+	-	-	-	-	-	-	-	-
	22	+	+	+	+	-	+	++	+	-	+	++	+

Suspensions of rabbit sperm cells in DM, BWW, T6, or TOY were incubated at 37°C in an atmosphere of 5% CO_2, 95% air. Sperm motion characteristics were observed under negative phase at 100x.

4. DISCUSSION AND CONCLUSION

It is clear that either T6 or TOY would be the medium of choice for the incubation of rabbit sperm cells. Sperm cells with hyperactive motion appeared after 3-5 hr and were also found in suspensions that were incubated for 22 hr in either medium. Sperm cells that did not develop hyperactive motion in DM often did so when incubated in either T6 or TOY. Because motility characteristics of sperm cells are relatively consistent within a rabbit, but different among rabbits, it is important to perform experiments with sperm cells from a single semen sample and not mix semen from rabbits. The recommended conditions for culture of rabbits' sperm cells are after removal of seminal contaminants by centrifugation through a Percoll gradient, incubation at 37 °C under an atmosphere of 5% CO_2 and 95% air as a suspension in either T6 or TOY. Preincubation in high ionic strength (HS), T6, or TOY mediums is not necessary.

The inability of rabbit serum to support sperm motility for more than 3-5 hr and maintain hyperactive motion was unexpected. The present experiments used a commercially available serum (GIBCO), which consisted of mixed sera obtained from males and females. Experiments reported by Akruk, Humphreys, and Williams,[5] and Viriyaphanich and Bedford[6] used sera from superovulated does to prepare defined medium (DM) for rabbit sperm capacitation. It is likely that a factor(s) is present in serum of superovulated does that is essential for the maintenance of motility and hyperactive motion. Although cells with swimming motions characteristic of hyperactive sperm are present after incubation in either T6 or TOY, it is not clear if these cells are capacitated and are able to fertilize an egg. Nevertheless, sperms cells that are not hyperactive are unable to fertilize an egg, and compounds tnat prevent the acquisition of hyperactive motion will inhibit fertilization. Rabbit sperm cells incubated in either T6 or TOY can be used for the in vitro assessment of the potential antifertility affects of compounds.

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